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Time-resolved autoantibody profiling facilitates stratification of preclinical type 1 diabetes in children

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Time-resolved autoantibody profiling facilitates stratification of preclinical type 1 diabetes in children

Short running title: Modeling of longitudinal autoantibody profiles

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Abstract

Progression to clinical type 1 diabetes varies between children developing beta-cell autoantibodies. Differences in autoantibody patterns could relate to disease progression and etiology. Here we modeled complex longitudinal autoantibody profiles using a novel wavelet-based algorithm. We identified clusters of similar profiles, associated with different types of progression, among 600 children from The Environmental Determinants of Diabetes in the Young birth cohort study who developed persistent autoantibodies against insulin (IAA), GAD (GADA) and/or insulinoma-associated antigen-2 (IA-2A), and were followed prospectively in 3 to 6 months intervals (median follow-up 6.5 years). Among multiple autoantibody-positive children (n=370), progression from seroconversion to clinical diabetes ranged between clusters from 6% (95%CI [0, 17.4]) to 84% (59.2, 93.6) within 5 years. Highest diabetes risks had children who seroconverted early in life (median age <2 years) and developed IAA and IA-2A that were stable-positive on follow-up, and these risks were unaffected by GADA status. Clusters lacking stable-positive GADA responses showed higher proportions of boys and lower frequencies of the HLA-DR3 allele. Our novel algorithm allows refined grouping of beta-cell autoantibody-positive children with distinct progression to clinical type 1 diabetes and provides new opportunities in searching for etiological factors and elucidating complex disease mechanisms.

Clinical type 1 diabetes is commonly preceded by the development of autoantibodies against pancreatic beta-cell antigens such as insulin autoantibodies (IAA), GAD autoantibodies (GADA), insulinoma-associated antigen-2 autoantibodies (IA-2A), and zinc transporter 8 autoantibodies (ZnT8A) (1). In particular, children who have developed two or more of these autoantibody types almost inevitably progress to clinically symptomatic diabetes (2). These findings have led to a new staging of type 1 diabetes, which classifies the presence of advanced beta-cell autoimmunity (multiple autoantibodies) in individuals without diabetic symptoms as an early stage of disease, i.e. presymptomatic type 1 diabetes (3,4). However, the time of progression from presymptomatic to clinical type 1 diabetes varies within multiple autoantibody-positive children (2). Autoantibody characteristics are known to stratify diabetes risk, including the age of seroconversion (2,5-7), antibody number (8-10), titer (6,7,9-12), affinity (13,14), antigen specificity (9,15-17) and epitope binding (9,14,18,19). Nevertheless, the relationship between various longitudinal autoantibody profiles and the rate of progression to diabetes remain yet rarely studied. The Environmental Determinants of Diabetes in the Young (TEDDY) study recently reported that among multiple autoantibody-positive children, those who reverted from GADA-positive to GADA-negative status on follow-up had greater diabetes risk than those with persistent autoantibodies (20). Likewise, clustering of children based on similarities between sequential autoantibody patterns in the German BABYDIAB cohort revealed delayed progression to type 1 diabetes in multiple autoantibody-positive children who became IAA-negative on follow-up (21). However, no study to date has analyzed longitudinal profiles of multiple autoantibodies in due consideration of the timing of changes in qualitative status of the different autoantibodies.

The TEDDY study provides unique opportunities for the analysis of longitudinal autoantibody profiles based on whole time series of autoantibody sequences due to frequent sampling and measurements of type 1 diabetes-associated autoantibodies starting in early infancy (22). This

could refine stratification of progression to clinical diabetes based on similarities in the timing of changes in autoantibody responses. However, the high complexity and multivariate nature of the longitudinal autoantibody data remains a challenging task for analysis. To address this issue, we developed a mathematical algorithm based on Haar wavelet decomposition that enables clustering of children according to similarities in their longitudinal autoantibody profiles. In contrast to most published approaches (2,5-10,12,20), our proposed method does not require a priori definition of relevant autoantibody patterns or seroconversion ages but intrinsically groups children taking longitudinal characteristics into account.

Research Design and Methods

Study population and samples The TEDDY study is a prospective cohort study with the primary goal of identifying environmental causes of type 1 diabetes. It includes six clinical research centers, three in the USA (Colorado, Georgia/Florida, Washington) and three in Europe (Finland, Germany, Sweden). Detailed study design and methods have been published previously (22). TEDDY enrolled 8,676 children who are genetically at-risk for developing type 1 diabetes based on HLA genotypes (23). Children enrolled are monitored prospectively from age 3 months to 4 years with study visits every 3 months and thereafter every 3 or 6 months until age 15 years, depending on autoantibody positivity. Children who are persistently positive for any autoantibody are monitored every 3 months until the age of 15 years or onset of type 1 diabetes. If remission of all autoantibodies occurs for a period of 4 consecutive visits or 1 year, an interval of 6 months becomes effective. Autoantibody-negative children are monitored every 6 months. The study was approved by local Institutional Review or Ethics Boards and monitored by an External Evaluation Committee formed by the National Institutes of Health. All participants provided written informed consent before participation in the genetic screening and in the prospective follow-up.

As of 31 December 2014, 618 children had developed persistent confirmed beta-cell autoantibodies (IAA, GADA and/or IA-2A; 242 single autoantibody-positive, 376 multiple autoantibody-positive) during a median follow-up of 6.5 years (IQR 5.2-8.0 years), and 172 of those had developed diabetes. To avoid bias due to short follow-up profiles, all children with less than five longitudinal samples were excluded from the analysis (n=18). Thus, the current analysis included 600 children (230 single autoantibody-positive and 370 multiple autoantibody-positive), and 165 of those developed diabetes. We analyzed the qualitative status of IAA, GADA and IA-2A over time using 37,047 measurements from birth.

Beta cell autoantibodies IAA, GADA, and IA-2A were measured in two laboratories by radiobinding assays, as previously described (22). In the U.S., all sera were assayed at the Barbara Davis Center for Childhood Diabetes at the University of Colorado Denver; in Europe, all sera were assayed at the University of Bristol, U.K. Both laboratories reported high sensitivity, specificity, and concordance (24). All positive beta-cell autoantibodies and 5% of negative samples were retested in the other reference laboratory and deemed confirmed if concordant. Persistent beta-cell autoimmunity was defined as autoantibody presence on two or more consecutive visits 3 months apart and confirmed in two TEDDY laboratories. Age of seroconversion was defined as the age of the child on the initial date of seroconversion to persistent beta-cell autoimmunity, as previously described (25). A child was considered multiple autoantibody-positive if at least two autoantibodies of IAA, GADA and/or IA-2A were positive in two consecutive samples or if at least two of these autoantibodies were positive in the last available sample prior to the development of type 1 diabetes. An autoantibody response was defined as transiently positive if at least two consecutive samples were autoantibody-positive and then followed by at least two consecutive autoantibody-negative samples or by an autoantibody-negative last available sample. An autoantibody

response was defined as stable-positive if it was not transiently positive. An autoantibody profile was defined as the qualitative status of IAA, GADA, IA-2A (i.e. positive/negative defined by cut-off) at a single time point, building a three-dimensional binary vector. A longitudinal autoantibody profile was defined as the temporal sequence of all single autoantibody profiles of a child. Type 1 diabetes was defined according to American Diabetes Association criteria for diagnosis (3).

Statistical Analysis Based on binary longitudinal autoantibody profiles of IAA, GADA and IA-2A (i.e. temporal sequences of positive or negative autoantibody status of all children), we developed a mathematical algorithm using Haar wavelets (26) to quantify the similarity between longitudinal autoantibody profiles. Subsequently, hierarchical clustering was performed to group children based on similarities. Imputation of data was required whenever samples were missing in the sequence of autoantibody measurements. A missing sample was assigned autoantibody-positive if the last prior sample and the next subsequent sample were positive for the particular autoantibody. In all other cases, missing samples were assigned autoantibody-negative.

Follow-up time and, accordingly, the number of available samples and autoantibody measurements varied considerably between children. We observed a bimodal distribution of follow-up time (Suppl. Fig. 1), with 82 children (69 multiple and 13 single autoantibody-positive) being followed for up to 42 months and 518 children (301 multiple and 217 single autoantibody-positive) being followed for more than 42 and up to 122 months, respectively. Since only shared follow-up periods could be used for pairwise comparison of children, short periods that were shared between children with considerably different lengths of follow-up did not contain sufficient information to achieve reasonable clustering results based on wavelet coefficients alone, as children with qualitatively different longitudinal autoantibody

profiles could be clustered together. We therefore first grouped children with long (more than 42 months) or short (up to 42 months) follow-up periods separately and later integrated the children with short follow-up into the clusters of children with long follow-up by using a combination of similarity of autoantibody patterns and similar timing of autoantibody development.

At first, a Haar wavelet decomposition was applied to autoantibody sequences of children with more than 42 months of follow-up, separately for IAA, GADA, and IA-2A. The resulting wavelet coefficients of the three autoantibodies were then combined into a single vector. The latter was used to estimate Euclidean distances of wavelet coefficients between pairs of children. Hierarchical clustering with complete linkage was performed on the resulting distances, separately for multiple autoantibody-positive and single autoantibody-positive children, respectively. At second, children with shorter follow-up of up to 42 months were assigned to clusters of children with longer follow-up profiles by using a combination of distances based on wavelet coefficients and a recently described algorithm for measuring similarity between sequential autoantibody patterns (21). For both measures, a Student's *t* statistic was calculated to compare the distances of children from each cluster to distances of children from all other clusters. Children with short follow-up were assigned to clusters of children with long follow-up with the maximum of the sum of both measures. The combination of distances from wavelet coefficients and from sequential autoantibody patterns ensured that children with short follow-up times were assigned to clusters with both similar timing of autoantibody appearance and similar longitudinal autoantibody profiles.

Kaplan-Meier survival analysis with log-rank test was used to compare the progression from autoantibody seroconversion to type 1 diabetes between clusters. The time from the age of seroconversion to the age at diagnosis of diabetes or the age at last contact in non-diabetic children was used as event time. Analysis considered censoring for losses to follow-up. The

5-year diabetes-free survival is presented for clusters comprising 10 or more children. Fisher's exact test was used to compare frequencies between groups. All statistical analyses were performed using R version 3.2.2.

Results

Clustering of multiple autoantibody-positive children We hypothesized that clustering of multiple autoantibody-positive children based on their consecutive profiles of IAA, GADA, and IA-2A could provide refined stratification with respect to progression to clinical type 1 diabetes and disease etiopathogenesis, respectively. Clustering based on wavelet coefficients was performed for 370 children who developed multiple beta-cell autoantibodies. The resulting dendrogram (Fig. 1) was used to define 12 multiple autoantibody clusters (mC1-mC12) comprising groups of 12 to 88 children who differed with respect to the age of autoantibody appearance and/or autoantibody profiles on follow-up (Fig. 2). Characteristics of the children in these clusters are summarized in Table 1. The clusters differed considerably with respect to the progression of children from seroconversion to clinical diabetes, ranging from 6% (95%CI [0, 17.4]; cluster mC9) to 84% (59.2, 93.6; mC5) within 5 years (Table 1). In particular, those clusters with the shortest distance to each other in the dendrogram (e.g. mC7 and mC8; Fig. 2) had markedly different diabetes-risks, indicating that the approach could distinguish children with different progression based on relatively small differences in their longitudinal autoantibody profiles. Next, we explored whether the clusters could stratify progression in children with common characteristics such as similar seroconversion age and autoantibody patterns.

Children with seroconversion at very young age. First, we compared clusters of children with similar young age of seroconversion but variable longitudinal autoantibody profiles with respect to differences in their progression to clinical type 1 diabetes. We therefore selected all

clusters with median age of seroconversion <2 years. This resulted in six clusters (Fig. 3A) characterized by the development of either three stable-positive autoantibodies (cluster mC6), or two stable-positive autoantibodies in combination with a transiently positive or negative third autoantibody (clusters mC5, mC10, mC12), or stable-positive GADA (cluster mC11) or IA-2A (cluster mC3) in combination with a transiently positive or negative second and/or third autoantibody, respectively. Regardless of GADA status, children with the combination of stable-positive IAA and IA-2A (mC6 and mC5) had similar 5-year diabetes risks, being significantly higher than the risks in all remaining clusters of children with very young seroconversion age ($P<0.0001$, HR 2.8 (1.9-4.2); Fig. 3B, Table 1). In contrast, the 5-year diabetes risks were not significantly different between clusters of children with the combination of stable-positive GADA and IA-2A (mC10) or IAA and GADA (mC12) and those with just stable-positive GADA (mC11) or IA-2A (mC3), respectively (Fig. 3B, Table 1). However, the overall frequency of diabetes throughout follow-up was higher in clusters with stable-positive IA-2A (mC10 [63%] and mC3 [50%]) as compared to those without (mC12 [32%] and mC11 [21%]; $P=0.002$; Fig. 3C).

Children with similar autoantibody patterns. Second, we compared clusters of children with similar autoantibody patterns but variable age of seroconversion with respect to differences in their progression to clinical type 1 diabetes. We therefore grouped clusters based on autoantibody patterns over time, and then compared clusters within groups according to the median age of seroconversion at <2 years, 2-4 years and >4 years, respectively. This resulted in four groups of three clusters each (Fig. 4A, Suppl. Fig. 2A). Clusters were characterized by the development of either stable-positive IAA, GADA and IA-2A (clusters mC6, mC7, and mC2); stable-positive IA-2A and IAA, or, stable-positive IA-2A alone (clusters mC5, mC8, or mC3, respectively); stable-positive IA-2A and GADA (clusters mC10, mC9, mC4); or stable-positive GADA and IAA, or stable-positive GADA alone (clusters mC12, or mC11, and mC1,

respectively). Within each cluster group, younger age at seroconversion was generally associated with increased 5-year diabetes risk (Fig. 4B-E); with the exception of children in cluster mC3 who seroconverted at median age <2 years, developed stable-positive IA-2A, but lost IAA reactivity on follow-up (Fig. 2, Fig. 4A) and presented with relatively delayed progression to clinical diabetes (Fig. 4C). The most significant effects of younger seroconversion age on diabetes risk were observed among children developing three stable-positive autoantibodies (mC6 vs. mC7/mC2, $P<0.0001$, HR 5.4 [2.5-11.9]; Fig. 4B), those developing stable-positive IA-2A and IAA (mC5 vs. mC8, $P=0.02$, HR 2.3 [1.1-4.9]; Fig. 4C), and those developing stable-positive IA-2A and GADA (mC10 vs. mC9/mC4, $P=0.045$, HR 3.9 [1.0-9.3]; Fig. 4D). Clusters of children with seroconversion at median age <2 years also showed higher overall frequencies of diabetes compared to those with similar autoantibody patterns but older seroconversion age (mC6 vs. mC7/mC2, $P<0.0001$; mC5 vs. mC8, $P=0.007$; mC10 vs. mC9/mC4, $P<0.0001$; Suppl. Fig. 2B). In contrast, the 5-year diabetes risks and overall diabetes frequencies were not statistically different between children who mainly lacked IA-2A and developed stable-positive GADA (mC12 vs. mC11 vs. mC1, $P>0.05$ for all pairwise comparisons; Fig. 4E, Suppl. Fig. 2B), and this was irrespective of seroconversion age. Of note, the differences in diabetes risk and overall diabetes frequency between clusters of multiple autoantibody-positive children were not explained by ZnT8A status (Suppl. Fig. 2C).

Features associated with autoantibody patterns. Clusters lacking stable-positive GADA responses (clusters mC5, mC8, mC3) showed higher proportions of boys ($P=0.002$; Fig. 5A) and lower frequencies of the HLA-DR3 allele ($P=0.0002$; Fig. 5B) compared to all other multiple autoantibody clusters.

Clustering of single autoantibody-positive children To analyze characteristics associated with different patterns of autoantibody reactivity against single beta-cell antigens, 230 single autoantibody-positive children were clustered based on wavelet decomposition of longitudinal time series of IAA, GADA, and IA-2A. The resulting dendrogram (Suppl. Fig. 3) was used to define nine single autoantibody clusters (sC1-sC9) containing groups of 5 to 50 children who differed with respect to their longitudinal autoantibody profiles (Suppl. Fig. 4). Characteristics of the children in these clusters are summarized in the online appendix (Suppl. Table 1).

Children with stable-positive GADA responses were clustered into two groups (clusters sC1 and sC2) that differed with respect to the age of seroconversion ($P < 0.0001$; Suppl. Table 1) but had similar 5-year diabetes risks (Suppl. Fig. 5A,B). Compared to clusters sC1 and sC2 combined, significantly increased 5-year diabetes risk was observed for cluster sC5 consisting of children with stable-positive IAA ($P = 0.008$, HR 4.3 [1.3-13.5]; Suppl. Table 1, Suppl. Fig. 5A,B). None of the children in clusters characterized by either transiently positive GADA (clusters sC3 and sC6) or IAA (clusters sC7, sC8, sC9) developed diabetes on follow-up (Suppl. Table 1, Suppl. Fig. 5A,B). Clusters of children with transiently positive IA-2A were not observed.

Differences were seen in positivity for ZnT8A between clusters (Suppl. Table 1, Suppl. Fig. 5C). While 80% (4 of 5) of children in the small cluster sC4 (stable-positive IA-2A) developed ZnT8A, only 37% (15 of 41) and 12% (4 of 33) of children in clusters sC1 and sC2 (stable-positive GADA), and 10% (2 of 21) of children in cluster sC5 (stable-positive IAA) developed ZnT8A, respectively.

HLA genotype was associated with single autoantibody clusters. Of note, HLA DR3-DQ2/DR3-DQ2 was absent in clusters of children with stable-positive IA-2A (sC4) or IAA (sC5). In contrast, HLA DR3-DQ2/DR3-DQ2 was relatively frequent among clusters of children with stable-positive GADA (sC1 38%, sC2 24%) or transiently positive GADA (sC3

44%, sC6 24%; Suppl. Table 1, Suppl. Fig. 5D). Clusters with stable-positive GADA (sC1 and sC2) showed increased frequencies of the HLA-DR3 allele compared to clusters with stable-positive IAA (sC5, $P=0.003$; Suppl. Fig. 5D) or IA-2A (sC4, $P=0.05$; Suppl. Fig. 5D), respectively.

Discussion

In the current study, we have tackled the challenge of combined analysis of complex longitudinal profiles of multiple biomarkers, namely three different types of beta-cell autoantibodies, in a time-resolved fashion. Specifically, we considered the age and sequence of changes in the qualitative status (i.e. positive or negative) of each autoantibody in each serum sample collected throughout follow-up of 600 children who developed persistent confirmed IAA, GADA and/or IA-2A while participating in the TEDDY study, comprising more than 37,000 antibody measurements. Using a novel wavelet-based algorithm we were able to define similarities between the longitudinal autoantibody profiles of children, including the temporal resolution of changes in autoantibody patterns. Based on these similarities, we could then perform hierarchical clustering of single and multiple autoantibody-positive children to define clusters that were associated with markedly different progression rates from seroconversion to clinical diabetes, particularly among those children with multiple autoantibodies, ranging from 6% to 84% within 5 years. Furthermore, we could pinpoint specific autoantibody patterns and characteristics related to different progression rates. We suggest that our approach holds great potential for refined explorations into the underlying etiology of different phenotypes of beta-cell autoimmunity.

Strengths of our study include the unique and well-defined cohort, and the use of an innovative analytical approach. The TEDDY study is the largest prospective study to date that follows genetically at-risk children for the development of beta-cell autoimmunity and type 1

diabetes (22). TEDDY has collected various possible exposures that could be important to the appearance and progression of beta-cell autoimmunity (27-32). Associations with genetic risk factors and age of appearance, type and levels of beta-cell autoantibodies, and progression to clinical type 1 diabetes have recently been reported (7,20,25,33-35). This current analysis adds to these previous studies in a new manner in that our novel approach is data-driven and considers changes in autoantibody characteristics at the time they occur.

We made use of Haar wavelet coefficients (26) to define similarities between longitudinal profiles of children. This approach holds a number of advantages for the analysis of prospective study data. However, it has not yet been used in prospective studies in type 1 diabetes. Wavelets enable a time-frequency type decomposition of time series data. Applying an iterative scheme, coefficients determined at the earlier steps capture ‘high-frequency’ information in the data such as on-and-off switches, whereas coefficients at later iteration steps allow identifying long-term trends in time series data. Wavelets are therefore a powerful tool to characterize dynamic temporal patterns in autoantibody progression. Still, intrinsic characteristics of the method need to be considered. Firstly, using Haar wavelets (i.e. a decomposition based on piecewise constant functions) might not provide the best orders of approximation, while at the same time being computationally very efficient. Nevertheless, for the type of data analyzed in this study, Haar wavelet coefficients turned out to capture the information well enough and no wavelets with higher order moments were needed. Secondly, in order to compare longitudinal autoantibody profiles of children, time series of differing lengths had to be cut down to the length of the shorter series. Thus, in particular when comparing a very short time series with a longer one, the comparison based on wavelets ignores a substantial part of the information provided by the longer series. We compensated for this deficiency in our analysis by combining wavelet decomposition with another qualitative algorithm provided previously (21). Thirdly, the method also requires that time

series have to be sampled equidistantly. While this is the case in the TEDDY study, other decompositions would have to be applied for scattered data.

We have focused our analysis on the group of multiple beta-cell autoantibody-positive children. Considerable differences exist between children at this presymptomatic stage of type 1 diabetes with respect to the time until clinical disease onset (2,7,36). A well-known risk factor for faster progression to clinical diabetes among autoantibody-positive individuals is young age of seroconversion (5-7,34). It is therefore remarkable that we could a) distinguish different rates of progression among clusters of multiple autoantibody-positive children (n=217) who all seroconverted at very young age, and b) link differences in progression to defined longitudinal autoantibody profiles. The highest risks were seen in 115 children who developed both stable-positive IAA and IA-2A responses early in life (clusters mC6 and mC5). Interestingly, high risk in those children was not influenced by the presence (mC6) or absence (mC5) of stable-positive GADA responses. On the contrary, risk was significantly lower for 102 children who seroconverted early and developed multiple autoantibodies but not stable-positive IAA and IA-2A. This is in line with our previous observation in the BABYDIAB cohort that losing IAA reactivity is associated with delayed progression to type 1 diabetes in multiple autoantibody-positive children (21). Amongst clusters of children with similar autoantibody patterns, younger age of seroconversion was associated with faster progression rate. An exception to this rule were children who developed stable-positive GADA but lacked IA-2A responses, and progressed relatively slowly regardless of seroconversion age.

Of note, in order to develop an autoantibody response to GAD that was stable-positive over time and, therefore, presumably relevant for the individual immune phenotype and disease pathogenesis, the majority of those children appeared to require HLA-DR3. Associations between HLA-DR and beta-cell autoantibody specificity have been reported (13,14,25,37-39).

In particular, the TEDDY study has recently demonstrated that the appearance of either IAA or GADA as the first autoantibody in children was strongly influenced by the presence of HLA-DR4 or, respectively, HLA-DR3 (25,35). Our current data suggest an influence of HLA genotype on longitudinal autoantibody profiles. Likewise, male sex has been associated with IAA only as the first autoantibody in children (35). We have observed now a predominance of boys for longitudinal autoantibody profiles lacking stable-positive GADA responses, which requires further attention.

As limitation to our study, longitudinal ZnT8A profiles could not be included in the current clustering analyses due to incomplete time series of ZnT8A measurements, which otherwise would have caused considerable reduction in sample size. However, we considered the overall ZnT8A status of each child in our analysis. As expected, this revealed that some children in the ‘single’ autoantibody clusters in fact had developed ZnT8A as second positive beta-cell autoantibody. The strongest effect was seen in the small cluster sC4, characterized by stable-positive IA-2A, in which four of five children (all male, carrying HLA-DR4) were ZnT8A-positive and two have progressed to clinical diabetes. This illustrates that certain low frequency immune patterns could be highly disease-relevant. With respect to longitudinal GADA patterns our study in children could underestimate their effect on diabetes-risk given that GADA is associated with older onset type 1 diabetes (40). Another limitation is that the study population is highly selected for HLA-conferred risk of type 1 diabetes (23). Validation in a study population that is not preselected, and cohorts of individuals seroconverting to beta-cell autoantibodies at older age is therefore necessary to ensure wider applicability of our observations.

Altogether, our data support the notion that the individual pattern of beta-cell autoantibodies, i.e. the pattern of main target autoantigens, as well as the timing of their appearance, dynamic and progression to diabetes is influenced by gene-environmental interactions. It is possible

that certain disease-promoting factors or conditions could act on genetically predisposed individuals only within certain age windows. Identifying such etiological factors could potentially pave the way for new prevention therapies, and we believe that our analytical approach could prove useful in that search.

In conclusion, our novel wavelet-based clustering algorithm allows refined grouping of multiple beta-cell autoantibody-positive children. The data-driven approach can identify groups of children with distinct progression to clinical type 1 diabetes and provides new opportunities in elucidating complex disease mechanisms.

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Contribution statement. All authors attest to meeting the International Committee of Medical Journal Editors uniform requirements for authorship by making substantial contributions to conception and design of this paper; acquisition, analyzing, and interpreting the data; drafting or revising the article for intellectual content; and giving final approval of the published version. P.A. and W.z.C. proposed the analysis. W.z.C. and D.E. developed the algorithm. D.E. performed the analysis. P.A., D.E., and W.z.C. interpreted the findings, and wrote the manuscript. E.B., M.R., W.A.H., J.-X.S., Å.L., J.T., K.V., A.J.K.W., L.Y., B.A., J.P.K., and A.-G.Z. contributed to the acquisition, analysis, or interpretation of data. J.P.K., Å.L., W.A.H., M.R., J.-X.S., J.T., A.-G.Z., and B.A. designed the TEDDY study, and reviewed and edited the manuscript for intellectual content. E.B., K.V., A.J.K.W., and L.Y. reviewed and edited the manuscript for intellectual content. P.A. and W.z.C. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1. Distribution of features among the multiple autoantibody clusters (mC1-mC12).

T1D, type 1 diabetes; C-Section, Caesarean section; IQR, interquartile range.

| Autoantibody Cluster | all | mC1 | mC2 | mC3 | mC4 | mC5 | mC6 | mC7 | mC8 | mC9 | mC10 | mC11 | mC12 |
|---|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| N | 370 | 35 | 30 | 12 | 21 | 27 | 88 | 27 | 24 | 16 | 30 | 19 | 41 |
| Age (years) of seroconversion median (IQR) | 2.0 (1.1-3.1) | 4.0 (3.0-4.7) | 4.2 (2.8-5.5) | 1.0 (0.8-2.0) | 7.2 (3.3-7.5) | 1.0 (0.8-1.1) | 1.4 (0.8-1.8) | 3.3 (3.0-3.9) | 2.4 (1.8-2.9) | 2.5 (2.3-3.0) | 1.7 (1.0-2.2) | 1.6 (1.1-2.0) | 1.5 (1.0-2.3) |
| T1D prevalence (%) | 41 | 11 | 17 | 50 | 14 | 85 | 66 | 7 | 46 | 12 | 63 | 21 | 32 |
| 5-year T1D risk % (95% CI) | 44 (38-49) | 12 (0-23) | 28 (0-49) | 51 (5-75) | 26 (0-49) | 84 (59-94) | 63 (51-72) | 9 (0-21) | 53 (21-72) | 6 (0-17) | 41 (20-57) | 22 (0-39) | 40 (19-56) |
| Maternal T1D (%) | 6 | 14 | 10 | 8 | 0 | 7 | 5 | 4 | 4 | 6 | 3 | 0 | 5 |
| HLA DR3/DR3 (%) | 7 | 9 | 3 | 8 | 5 | 0 | 3 | 4 | 0 | 31 | 17 | 21 | 7 |
| HLA DR3/DR4 (%) | 57 | 69 | 63 | 33 | 48 | 44 | 64 | 56 | 38 | 56 | 43 | 68 | 63 |
| HLA DR4/DR4 (%) | 18 | 17 | 23 | 33 | 14 | 15 | 10 | 26 | 42 | 13 | 27 | 0 | 15 |
| HLA DR4/DRx | 18 | 6 | 10 | 25 | 33 | 41 | 22 | 15 | 21 | 0 | 13 | 11 | 15 |
| ZnT8A (%) | 62 | 51 | 73 | 83 | 52 | 44 | 58 | 74 | 71 | 75 | 77 | 47 | 56 |
| C-Section (%) | 22 | 26 | 20 | 50 | 24 | 37 | 17 | 26 | 17 | 25 | 20 | 16 | 20 |
| Male (%) | 56 | 57 | 50 | 83 | 52 | 74 | 55 | 52 | 67 | 69 | 50 | 47 | 41 |

Figure Legends

Figure 1. Hierarchical clustering results for longitudinal autoantibody profiles of 370 children who developed multiple beta-cell autoantibodies. The dendrogram is divided into 12 multiple autoantibody clusters (mC1-mC12). Each column represents the follow-up time from birth for one child. The qualitative status of IAA, GADA and IA-2A, respectively, is indicated by color (red = antibody-positive; blue = antibody-negative) with respect to the age at antibody measurement.

Figure 2. Aggregated longitudinal profiles of IAA, GADA, and IA-2A are shown for children of multiple autoantibody clusters (mC1-mC12). For each cluster, the percentage of children positive for the respective autoantibody is indicated by color (white: 0% positive; red: 100% positive) with respect to age. The blue and green lines indicate the age until which >50% and >25% of children in the cluster were followed, respectively. Autoantibody profiles are plotted until only two children in the cluster remained in follow-up.

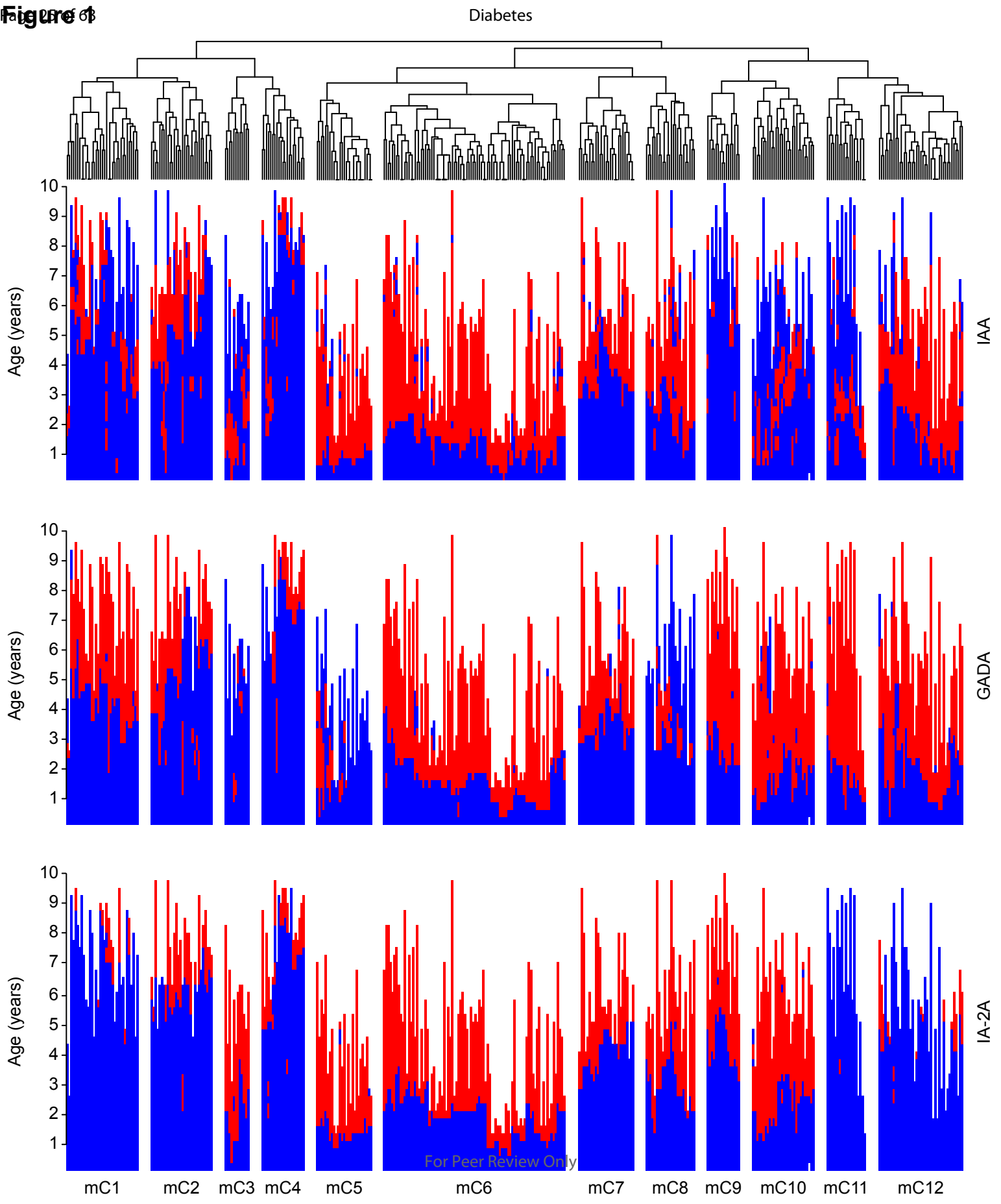
Figure 3. Characteristics of clusters of multiple autoantibody-positive children with seroconversion at very young age (median age <2 years). For each cluster is shown: **(A)** the percentage of children who were stable-positive, transiently positive, or negative on follow-up for IAA, GADA and IA-2A, respectively; **(B)** the cumulative diabetes-free survival from autoantibody seroconversion; **(C)** the overall frequency of diabetes throughout follow-up.

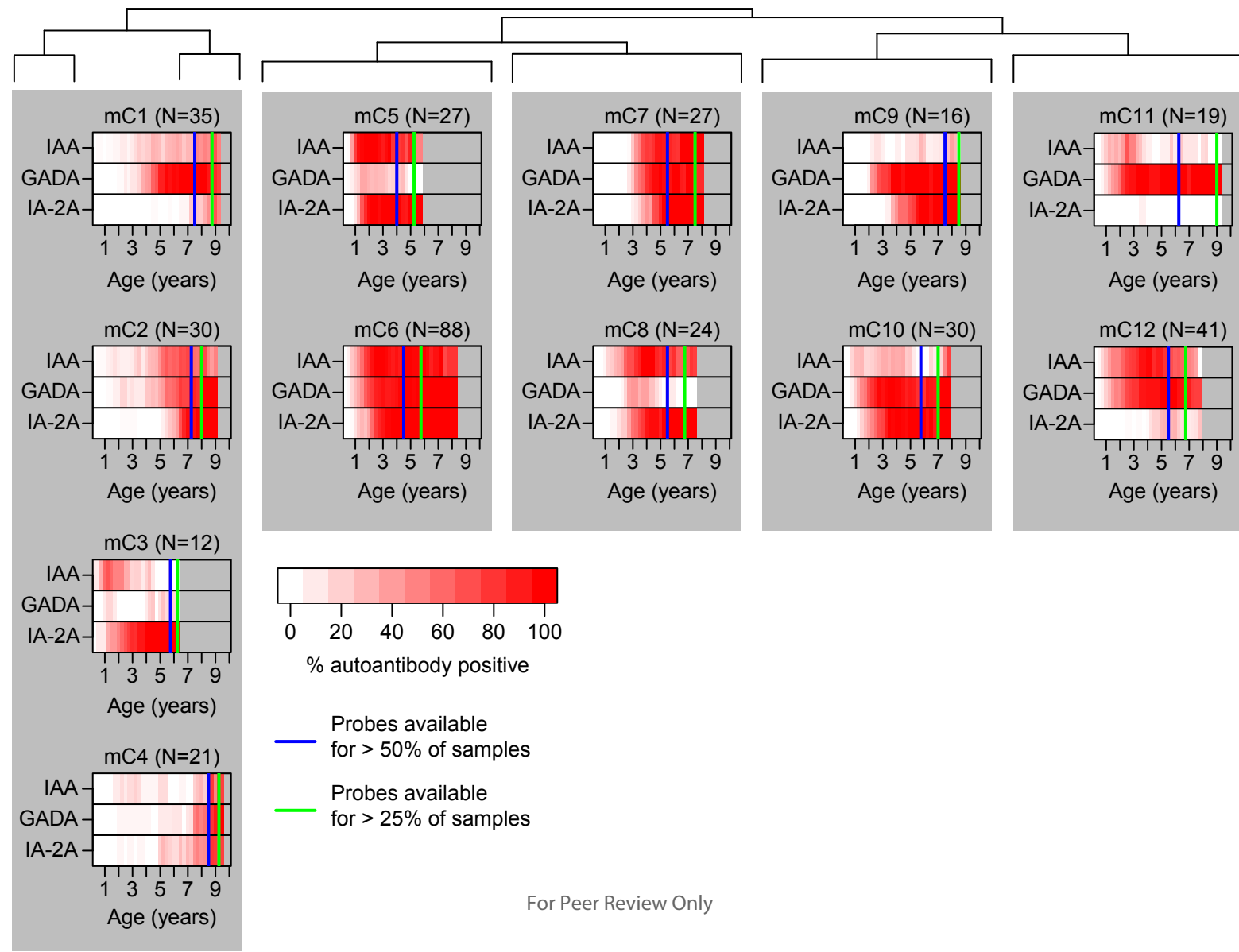
Figure 4. Progression to type one diabetes among clusters of multiple autoantibody-positive children with similar autoantibody characteristics but variable age of seroconversion. Clusters are grouped into four groups of three clusters each, based on similarity of autoantibody profiles. For clusters of each group is shown: **(A)** the percentage of children who were stable-

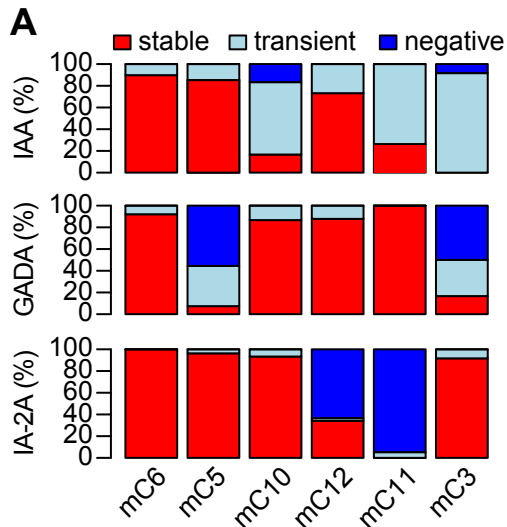
positive, transiently positive, or negative on follow-up for IAA, GADA and IA-2A, respectively; **(B-E)** the cumulative diabetes-free survival from autoantibody seroconversion.

Figure 5. The proportions of boys **(A)** and HLA-DR genotypes **(B)** are shown for multiple autoantibody clusters (mC1-mC12) grouped into four groups of three clusters each, based on similarity of autoantibody profiles. The cluster group mC5, mC8, mC3 comprised a significantly higher proportion of boys ($P=0.002$) and lower frequency of HLA-DR3 ($P=0.0002$) compared to the other cluster groups.

Figure 6

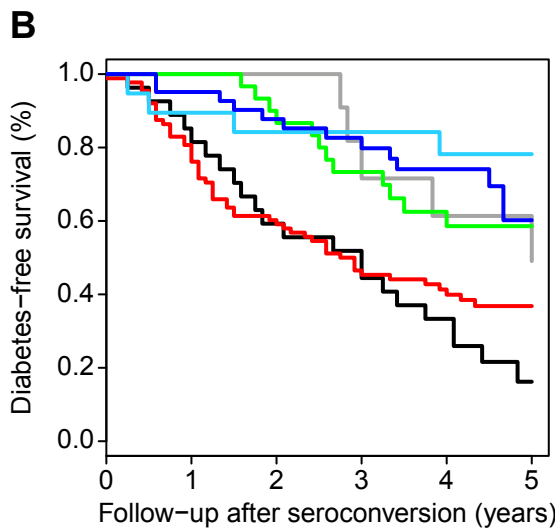






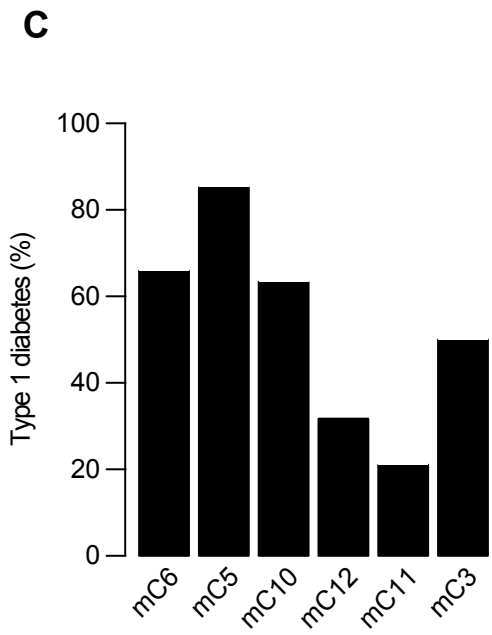
| Characteristic | Cluster | age (*) |
|---------------------------|---------|-----------|
| Stable IAA & GADA & IA-2A | mC6 | < 2 years |
| Stable IA-2A & IAA | mC5 | < 2 years |
| Stable IA-2A & GADA | mC10 | < 2 years |
| Stable GADA & IAA | mC12 | < 2 years |
| Stable GADA | mC11 | < 2 years |
| Stable IA-2A | mC3 | < 2 years |

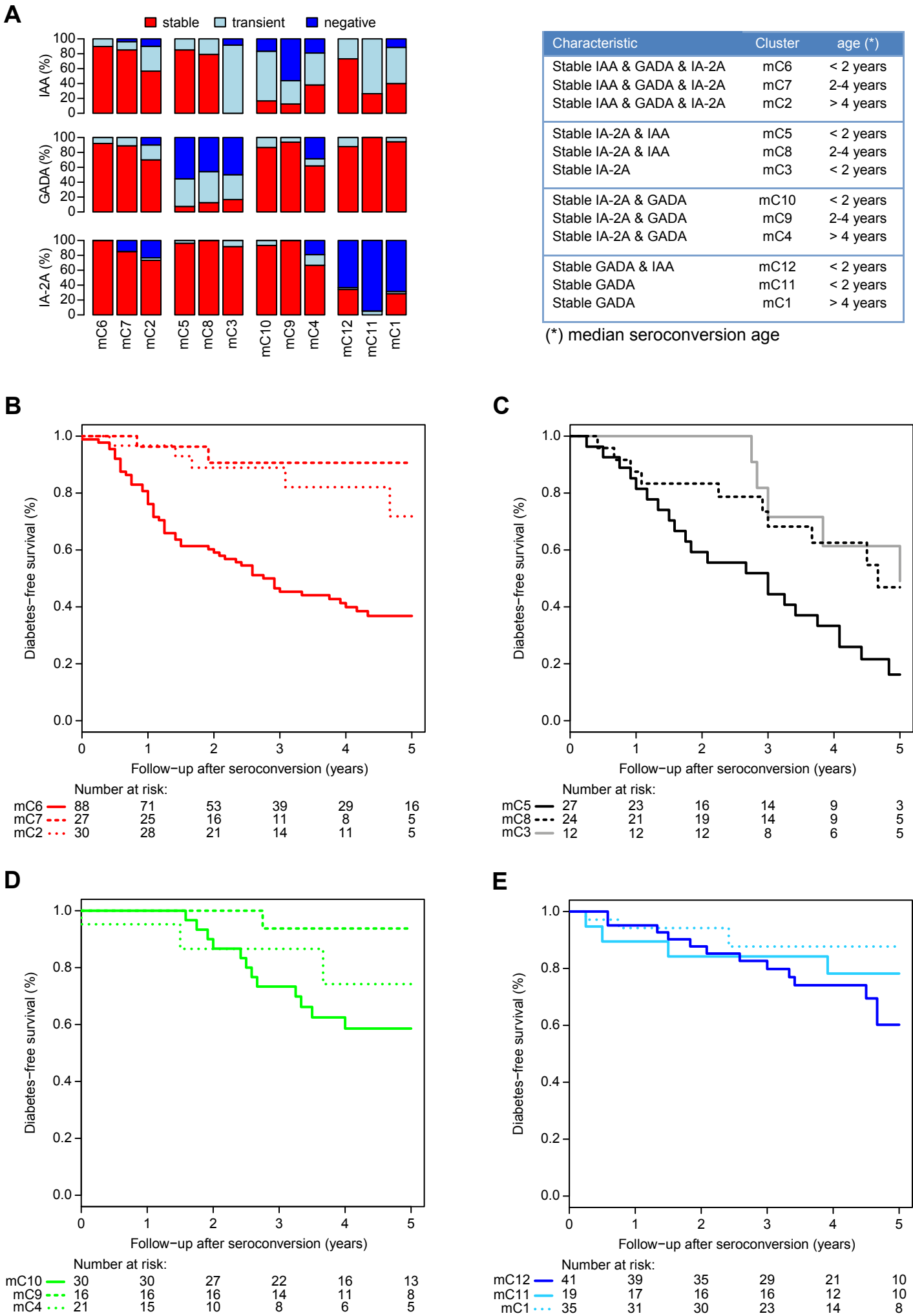
(*) median seroconversion age



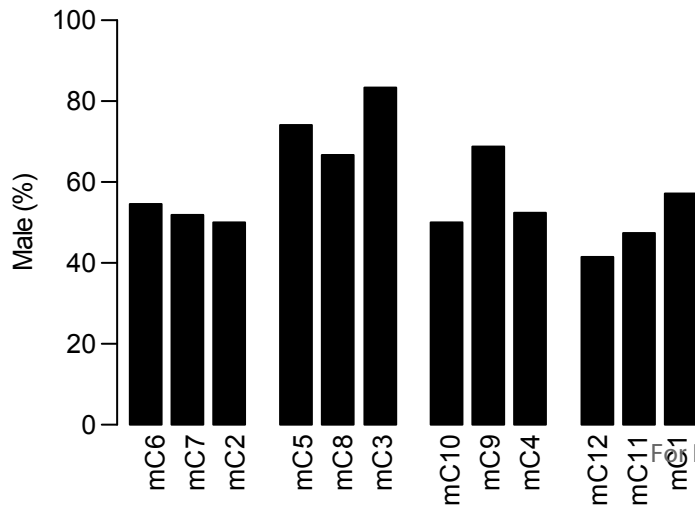
Number at risk:

| | | | | | | |
|------|----|----|----|----|----|----|
| mC6 | 88 | 71 | 53 | 39 | 29 | 16 |
| mC5 | 27 | 23 | 16 | 14 | 9 | 3 |
| mC10 | 30 | 30 | 27 | 22 | 16 | 13 |
| mC12 | 41 | 39 | 35 | 29 | 21 | 10 |
| mC11 | 19 | 17 | 16 | 16 | 12 | 10 |
| mC3 | 12 | 12 | 12 | 8 | 6 | 5 |



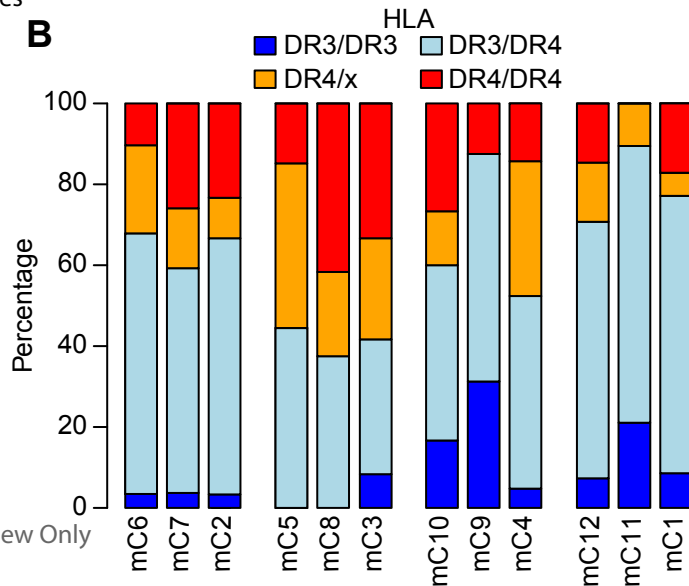


A



Diabetes

B



For Peer Review Only

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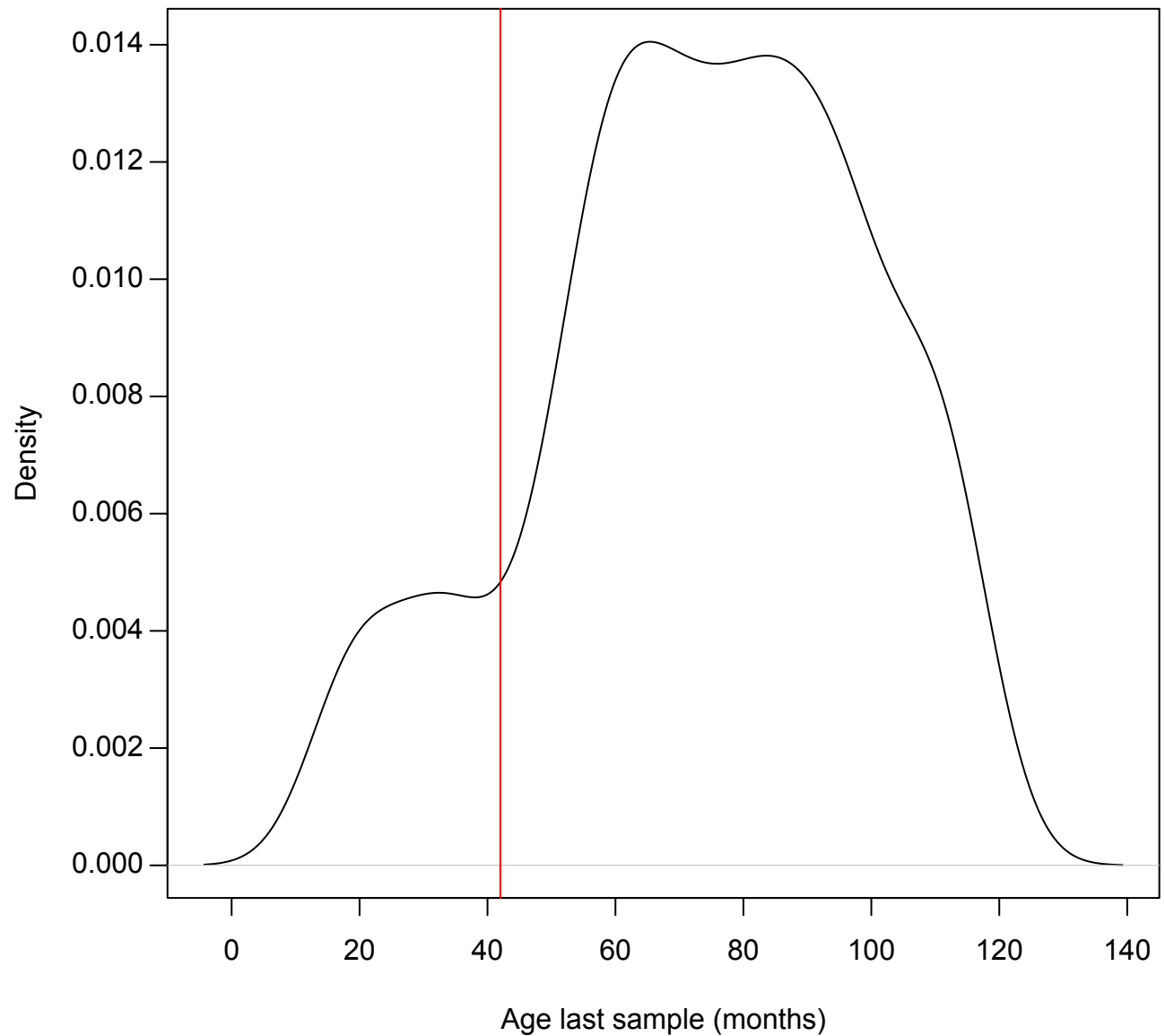
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Supplementary Material

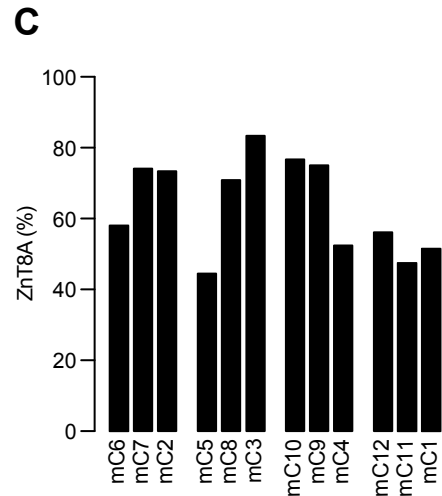
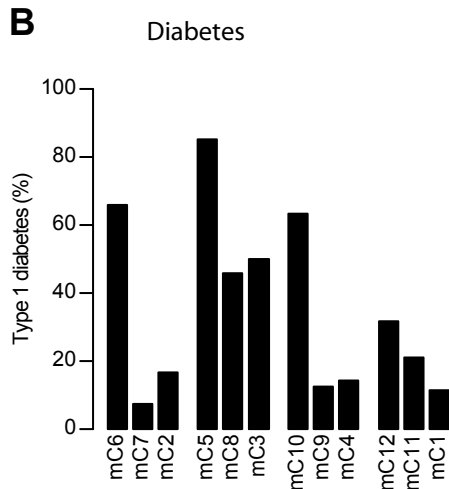
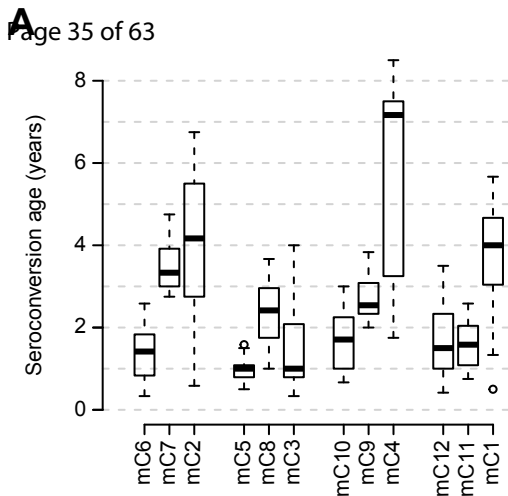
1. Supplementary Table 1
2. Supplementary Figure 1
3. Supplementary Figure 2
4. Supplementary Figure 3
5. Supplementary Figure 4
6. Supplementary Figure 5

Supplementary Table 1. Distribution of features among the single autoantibody clusters (sC1-sC9).
T1D, type 1 diabetes; C-Section, Caesarean section; IQR, interquartile range.

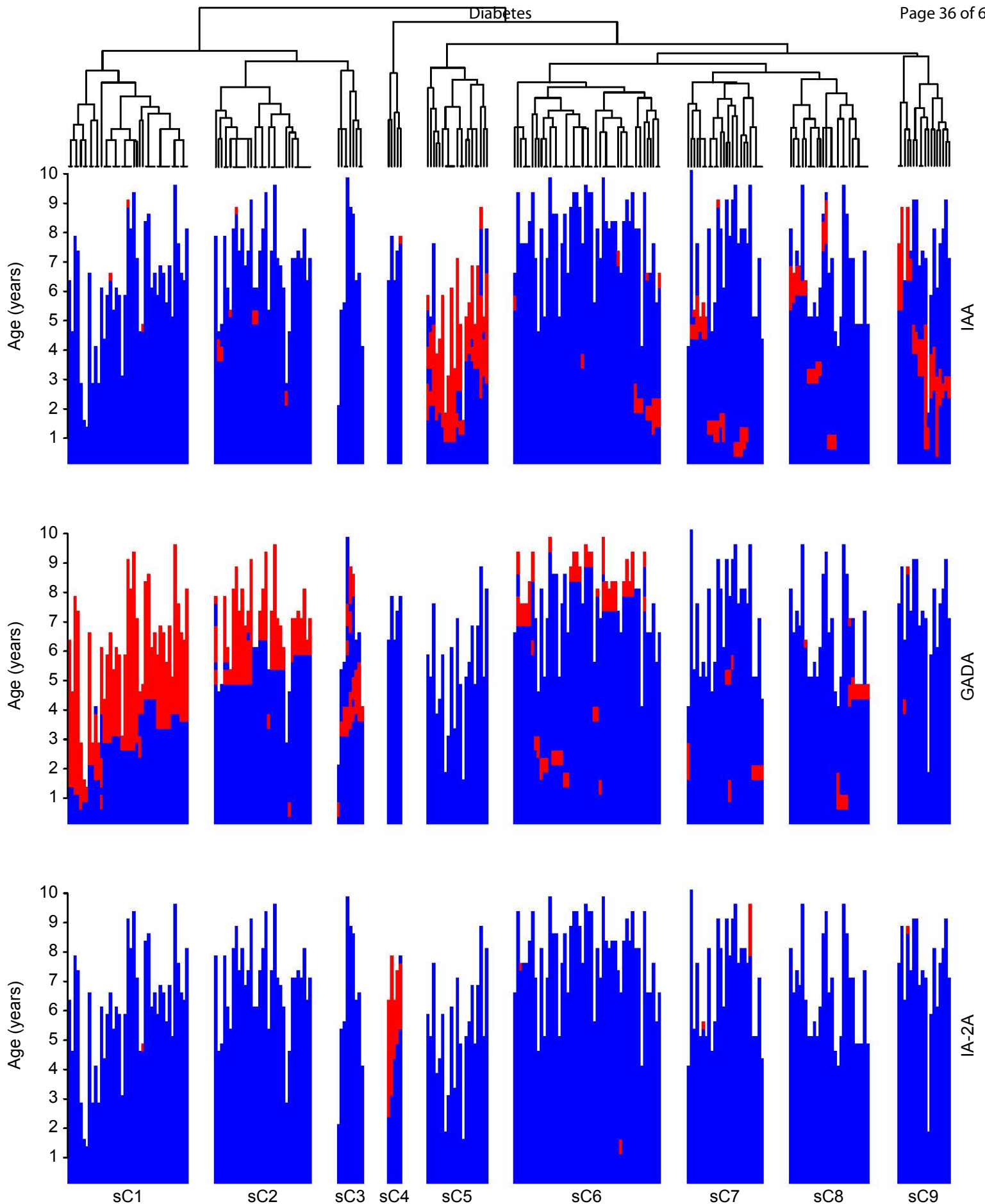
| Autoantibody Cluster | all | sC1 | sC2 | sC3 | sC4 | sC5 | sC6 | sC7 | sC8 | sC9 |
|---|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| N | 230 | 41 | 33 | 9 | 5 | 21 | 50 | 26 | 27 | 18 |
| Age (years) of seroconversion median (IQR) | 3.5 (1.8-5.1) | 3.1 (2.3-3.7) | 5.2 (5.0-6.0) | 3.5 (3.3-3.8) | 4.4 (3.3-4.8) | 2.1 (1.3-3.3) | 5.9 (2.0-7.7) | 1.8 (1.0-4.6) | 4.5 (1.9-5.7) | 3.1 (2.5-4.0) |
| T1D prevalence (%) | 7 | 12 | 3 | 0 | 40 | 33 | 0 | 0 | 0 | 0 |
| 5-year T1D risk % (95% CI) | 10 (4-15) | 12 (0-24) | 3 (0-90) | - - | - - | 46 (10-68) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) |
| Maternal T1D (%) | 4 | 5 | 3 | 0 | 20 | 5 | 4 | 4 | 0 | 6 |
| HLA DR3/DR3 (%) | 21 | 38 | 24 | 44 | 0 | 0 | 24 | 12 | 7 | 18 |
| HLA DR4/DR3 (%) | 42 | 43 | 45 | 33 | 40 | 52 | 34 | 54 | 44 | 29 |
| HLA DR4/DR4 (%) | 18 | 13 | 15 | 11 | 40 | 19 | 26 | 12 | 26 | 0 |
| HLA DR4/DRx | 20 | 8 | 15 | 11 | 20 | 29 | 16 | 23 | 22 | 53 |
| ZnT8A (%) | 14 | 37 | 12 | 0 | 80 | 10 | 6 | 15 | 0 | 6 |
| C-Section (%) | 26 | 27 | 30 | 44 | 40 | 24 | 26 | 15 | 22 | 22 |
| Male (%) | 57 | 49 | 70 | 44 | 100 | 62 | 62 | 65 | 52 | 22 |



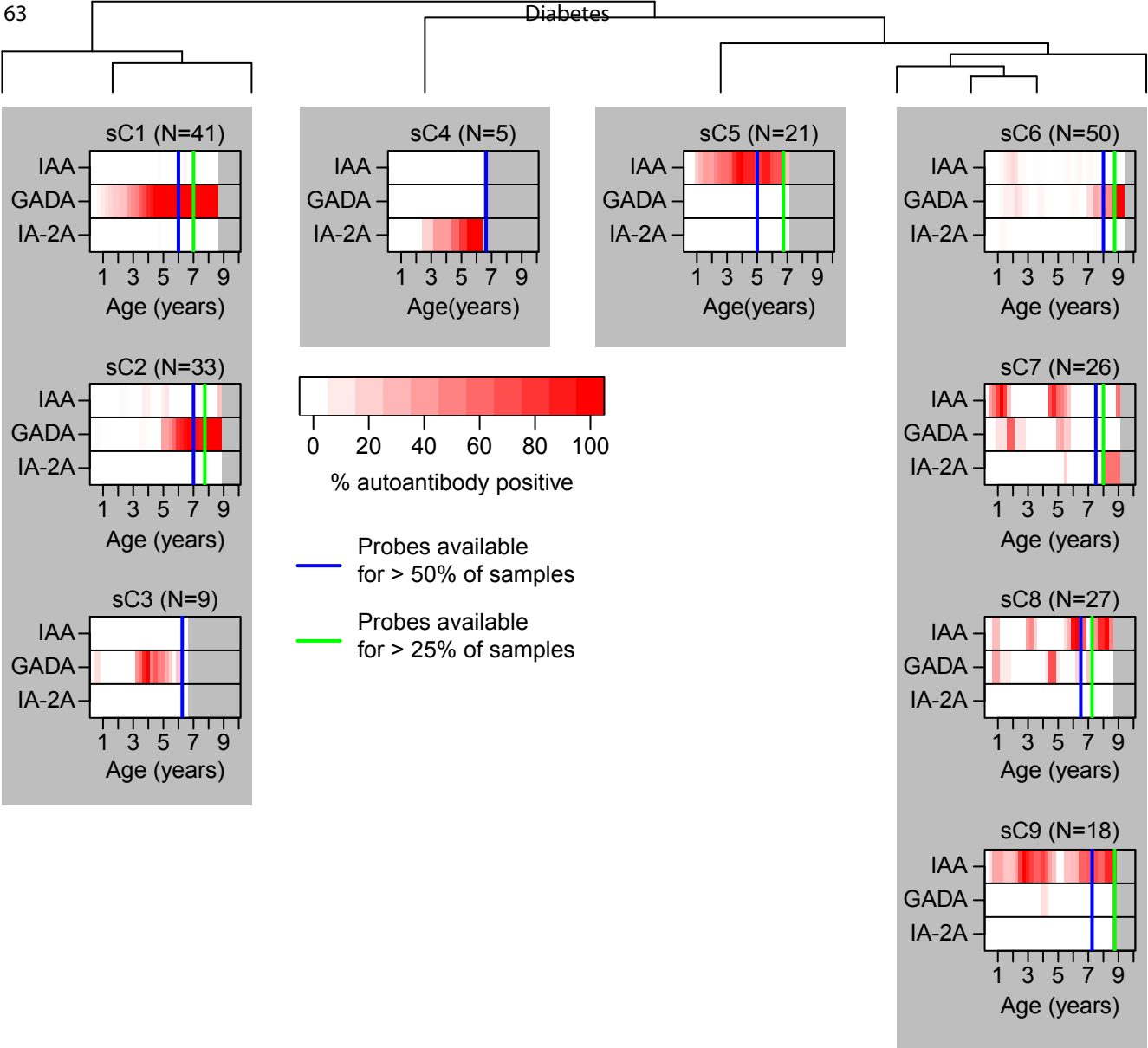
Supplementary Figure 1. Distribution of follow-up time of 600 children who developed persistent beta cell autoimmunity. Density is plotted for age of last available follow-up serum sample among all follow-up samples of all children. A vertical red line is drawn at age 42 months, separating two groups of children with short and long follow-up time, respectively.



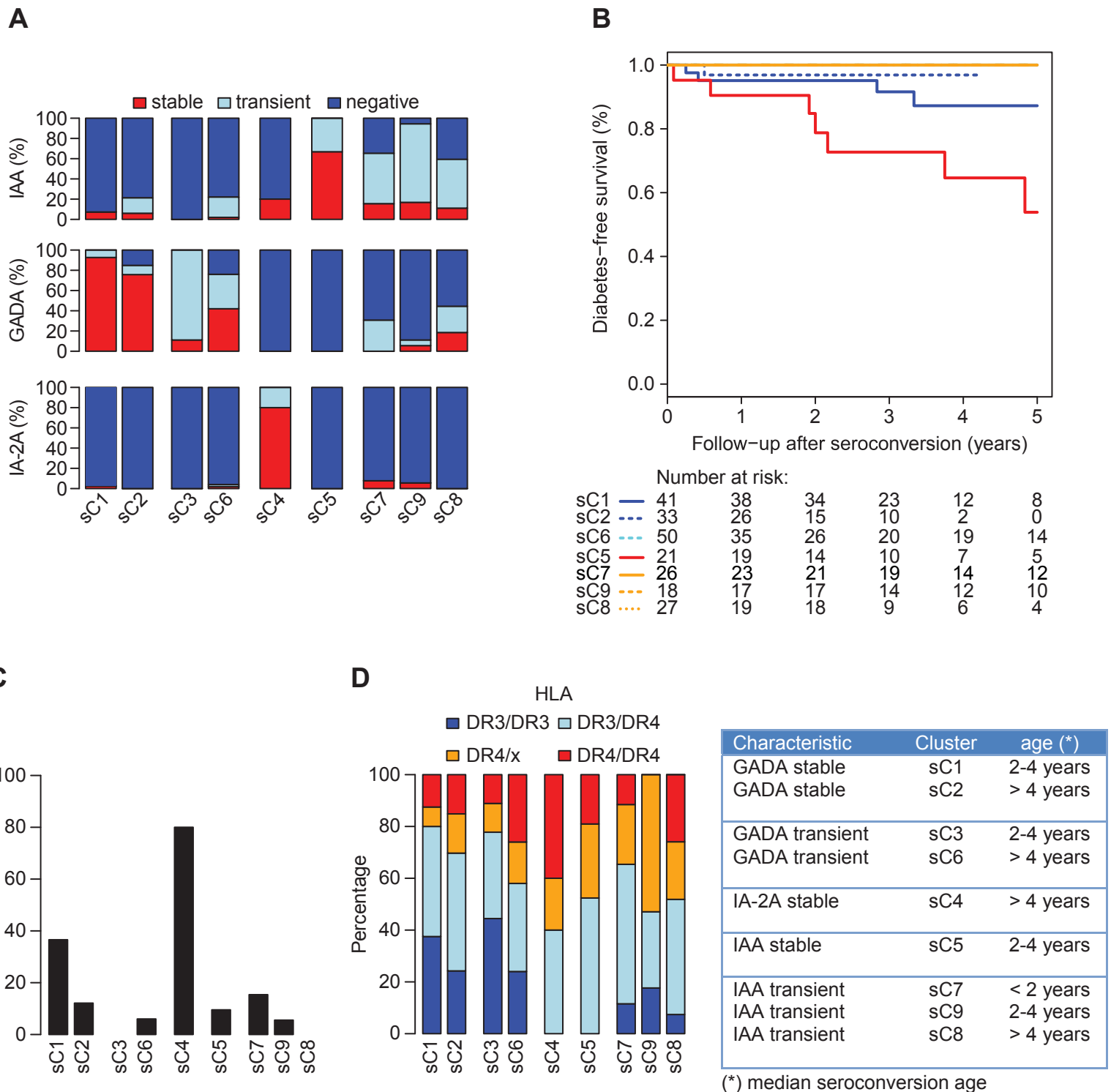
Supplementary Figure 2. Multiple autoantibody clusters (mC1-mC12) are grouped into four groups of three clusters each, based on similarity of autoantibody patterns over time. For clusters of each group is shown: **(A)** the age of seroconversion (median [quartiles]); **(B)** the overall frequency of diabetes throughout follow-up; **(C)** the percentage of children who were ZnT8A-positive on follow-up.



Supplementary Figure 3. Hierarchical clustering results for longitudinal autoantibody profiles of 230 children who developed single beta cell autoantibodies. The dendrogram is divided into 9 single autoantibody clusters (sC1-sC9). Each column represents the follow-up time from birth for one child. The qualitative status of IAA, GADA and IA-2A, respectively, is indicated by color (red = antibody-positive; blue = antibody-negative) with respect to the age at antibody measurement.



Supplementary Figure 4. Aggregated longitudinal profiles of IAA, GADA, and IA-2A are shown for children of single autoantibody clusters (sC1-sC9). For each cluster, the percentage of children positive for the respective autoantibody is indicated by color (white: 0% positive; red: 100% positive) with respect to age. The blue and green lines indicate the age until which >50% and >25% of children in the cluster were followed, respectively. Autoantibody profiles are plotted until only 2 children in the cluster remained in follow-up.



Supplementary Figure 5. Single-autoantibody clusters (sC1-sC9). For each cluster is shown: (A) the percentage of children who were stable-positive, transiently positive, or negative on follow-up for IAA, GADA and IA-2A, respectively; (B) the cumulative diabetes-free survival from autoantibody seroconversion (not shown for sC3 and sC4; each $n < 10$); (C) the percentage of children who were ZnT8A-positive on follow-up; (D) the proportions of HLA-DR genotypes. ¶